An-Najah National University
Faculty of Graduate Studies

In Vitro Regeneration and Somaclonal Variation of *Petunia hybrida*

By

Anas Yousef Abu-Rayya

Supervisor

Dr. Hassan Abu-Qaoud

Co-Supervisor

Dr. Sami Yaish

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Committee Decision

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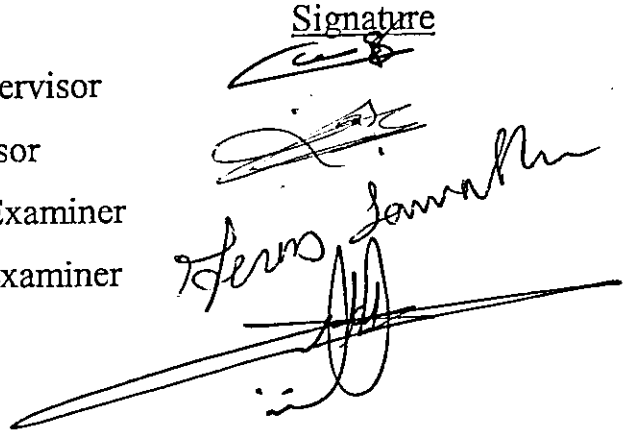
This thesis was defended successfully on the 11th March 2002 and approved by:

Committee Members	Signature
Dr. Hassan Abu-Qaoud –Supervisor	
Dr. Sami Yaish - Co-Supervisor	
Dr. Feras Sawalha -Internal Examiner	
Dr. Belal Ghareeb External Examiner	



DEDICATION

TO MY DEAR FATHER MOTHER AND WIFE

FOR THEIR SUPPORT

TO MY BROTHERS AND SISTER WITH LOVE

AND RESPECT

Acknowledgment

My sincere gratitude and appreciation to my supervisor Dr. Hassan Abu–Qaoud for his high experienced advices for achieving this thesis. I am greatly appreciative to Dr. Sami Yaish for reviewing the thesis.

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Abstract

In Vitro Regeneration and Somaclonal Variation of *Petunia Hybrid*

The effect of different levels of auxin Naphthaleneacetic acid (NAA) and cytokinin Benzyl adenin (BA) on shoot regeneration of *Petunia hybrida* was studied in these experiments. The regenerated shoots were grown for somaclonal variation. Seeds of *Petunia hybrida*_were in vitro planted onto MS basal medium. The growing seedlings were used as a source material for the regeneration experiments. Four levels of BA (0, 0.1, 0.4, 0.8 mg/l) and two levels of NAA (0.0, 0.1 mg/l) were used with MS basal media. Two sources of explants were used; leaf sections and shoots. Low regeneration percentage was achieved with leaf explants. The highest shoot number (1.7) was obtained with MS medium supplied with 0.4 mg/l BA and 0.1 mg/l NAA. However, in case of shoot explant higher shoot number was obtained with 0.8 mg/l BA and 0.1 mg/l NAA. For somaclonal variation, buds were taken from petunia plants of pink color that were grown in the green house. The buds were disinfected and grown on MS basal media supplied with 30 mg/l Gentamycin sulfate and 30 mg/l Benlate, after shoot growth they were transferred onto the shoot regeneration medium. The regenerates were rooted, acclimatized and transferred to the green house for evaluation. Two forms of leaf shape orbicular and elliptic and three flower colors violet, purple and light pink were appeared with the plants.

CHAPTER I
Introduction

1.1 General introduction

The world wide floriculture industry is estimated at four billion US dollars in 1991 with an annual increase of 8-10% (Hutchinson *et al*, 1992; Geneve *et al*, 1997). *Petunia hybrida* is an economically important (Encyclopedia, 2000) and commercially significant horticultural species, (Mol *et al.*, 1985; Quattrocchio *et al.*, 1993; 1998; Huits *et al.*, 1994; Solani *et al.*, 1995; Bradley *et al.*, 1998; Davies *et al.*, 1998)

Petunia have been diversified so greatly and made available in a range of colors, making it possible to select a plant whatever the color range that elaborate color schemes to be created using them alone (Christopher, 1994) so it is widely cultivated in many countries for its showy flowers (Encyclopedia, 2000).

Ornamental plants are produced exclusively for their esthetic values. Thus the improvement of quality attributes such as flower color, longevity and plant shape, architecture, (Burchi *et al.*, 1995), reduction of chemicals used for disease control (Jain and De Klerk, 1998), and creation of novel variation are important economic goals (Burchi *et al.*, 1995).

Further growth and success depends on the development of new technologies (Encyclopedia, 2000), such as tissue culture (Geneve *et al.*, 1997), which involves the production of plants

from tiny pieces of young plant material (Christopher, 1994) tissue or cells grown aseptically in test tube or other containers where the environment and nutrients can be rigidly controlled (Hudson & Dale, 1983).

The variability associated with tissue culture has provided a pool of variation upon which selection pressure has imposed to isolate unique forms of clones. This variation known as somaclonal variation, which becomes important for plant improvement (Skirvin *et al.*, 1993). Ornamental characteristics are suitable for improvement by mutation breeding and tissue culture technique (Hutchinson, *et al.,* 1992).

Therefore the aim of this investigation was to establish

1. *In vitro* regeneration system for *Petunia hybrida* plant.
2. *In vitro* multiplication media for the regenerates.
3. A rooting and acclimatization system.
4. *Ex vitro* evaluation of the regenerates.

1.2 Plant tissue culture

The ability to grow plant tissue, such as callus and cell and various plant organs has been utilized in scientific laboratories as a research tool for geneticists, botanists, and plant pathologists. The

methods used have been known collectively as tissue culture (Hudson & Dale, 1983).

Plant tissue culture originated at the turn of the 20th century, with the Haberlandt cell totipotency. He envisaged conversion of cells *en masse* to roots, shoots and embryo-like structures, thus permitting fundamental studies of plant development (Lowe, *et al.*, 1996).

The past twenty five years have seen a dramatic investment in tissue culture technology; the chemical and physical culture conditions have been refined for many species, with an infinite number of media permutations being reported (Lowe, *et al.*, 1996). Tremendous developments have taken place following the identification of indole-3-acetic acid (IAA) as auxin (Pierik, 1987) by Kogl *et al.*, (1934) and the discovery of kinetin (Murashige & Huang, 1987), the first cytokinin, by Miller *et al.*, 1956.

The difference between tissue culture and traditional methods for cloning involve the use of smaller space (George & Sherrington, 1984), the provision of artificial environment and aseptic conditions which reduce the risk of infection by pathogens (George & Sherrington; 1984 Kyte, 1987), thus producing plants of superior quality and better health. The plants can be rapidly cloned *in vitro* than *in vivo* (Pierik, 1987). Comparing the two

methods (Kyte, 1987), only one plant is produced from one seed by the conventional methods while one explant can produce an infinity number of plants. Pierik, 1987; Conger, 1981, reported that many years are required to get genetically uniform parent plants in large numbers for large scale hybrid seed production. In contrast, multiplication by tissue culture is quite rapid (Kyte, 1987).

Tissue culture systems have two major uses; primarily rapid mass propagation of clones, development, maintenance, and distribution of specific pathogen-tested (SPT) clones.

Secondarily, in vitro culture systems have a potential for long distance shipment of propagation material, as long-term storage of clonal material (Bajaj, 1979; Kartha, 1980 and Westcott, 1977) parallel to these propagation uses, tissue culture systems have a potential for production of various secondary production, such as pharmaceuticals in cell suspension systems (Bettell, 1979; Dougall, 1979; Gamborg, 1979 and Vasil, 1979).

There are many current technologies based on tissue culture such as virus elimination from plants (Lowe *et al.*, 1996), yielding high level of secondary products (Stafford, 1991), or using tissue culture as a plateform for genetic engineering approaches including transformation, insertion and expression of a specific

DNA sequences (Lowe *et al.*, 1996). A notable example of the importance of micropropagation is tulip. Since in the field each bulb produces two to three daughter bulbs per year, it takes about five years until sufficient bulbs have been produced for introduction of a new tulip genotype on the market by tissue culture, this period can be reduced by ten or fifteen years (Jain and Klerk, 1998).

Tissue culture is also used by researchers and growers to rapidly generate numerous clones year- round in greenhouses, and to propagate species which are difficult to grow commercially from cuttings, layering, or grafting (Encyclopedia, 2000).

In addition tissue culture is also used to overcome sexual incompatibility between wild species which contain a rich gene pool for desirable traits and cultivated crops to generate novel intra and intergeneric hybrid plants, at the cell level, occasional inter-familial hybrids (Hammatt, *et al.*, 1990).

1. 2. 1 Somaclonal variations

In floriculture, introduction of new cultivars on the market is essential: just like, in the fashion industry, new or unusual traits increase the demand. Furthermore, production of new cultivars is required to meet increasing environmental demands, in particular

the reduction of chemicals used for disease control (Jain & Klerk, 1998).

Plant regenerated from tissue cultures often display genetic (Braun, 1959) chromosomal and morphological variations that are not present in the parental materials (Engler and Grogan, 1984; Kim and Kim 1987 and Nelson, *et al.*, 1986). This phenomenon has been termed 'somaclonal variation (Larkin and Scowcroft, 1981). The source of this variation can result from genetic mutation, epigenetic change, or a combination of both. Genetic mutations involve random alternations in genetic constitutions, deletions, duplications, and rearrangements of genetic material.

The genetic change associated with somaclonal variation are point mutations, karyotype change (chromosome number, structure), cryptic changes associated with chromosome rearrangements, altered sequence copy number, transposable elements, somatic crossing-over, sister chromatid exchange, DNA amplification, and deletion (Karp, 1995; Brar and Jain, 1997).

Epigenetic changes primarily involve time or tissue specific selective gene expression rather than sorting out of genetic determinants (Meins, 1983).

Somaclonal variation may be epigenetic, but many examples point to genetic control; it may be related to genetic variation in

the original cells, which is realized during culture (Rice *et al.*, 1993), such somaclonal variation may be stable and heritable and has resulted in plants with agronomically useful traits (Karp, 1995, Larkin and Banks, 1995).

Tissue culture enables fast propagation (Jain & Klerk, 1998). Therefore, somaclonal variation is now known to be widespread among tissue culture derived plants (Skirvin *et al.*, 1993).

Thus, tissue culture can be considered as the simplest, low cost method for genetic manipulation (Lowe *et al.*, 1996).

1.2.2 Steps involved in the use of somaclonal variation in ornamental plant breeding

Steps involved in the utilization of somaclonal variation were summarized by Jain *et al.*, 1997 as follows:-

1- Induction and growth of callus or cell suspension cultures for several cycles.

2- Regeneration of large numbers of plants from such long-term cultures.

3- Screening for desirable traits in the regenerated plants and their progenies (ornamental characters are usually only visible in adult or flowering plants.

4- Testing of selected variants in subsequent generations for desired traits.

5- Multiplication of stable variants and/or use of them in breeding programs.

1.2.3 Factors affecting somaclonal variation

Various conditions during tissue culture influence the extent of somaclonal variation. The length of the culture period may be important. The reduction, and even the total loss of regeneration ability, is a general phenomenon observed during undifferentiated cell culture (Nehra *et al.*, 1992).

Somaclonal variation was also observed when somatic embryogenic cultures were kept for longer time in vitro conditions (Henry *et al.*, 1997). Hormone balance also may influence, probably indirectly, genome dynamics during culture (Bogani *et al.*, 1995, 1996).

The frequency of somaclonal variation strongly depends on the way of regeneration (Kawata and Oono, 1997 and Jain, 1997), plants regenerated from unorganized callus vary more than those from organized callus, whereas no or hardly any variation occurs when plants regenerated directly without an intermediate callus phase (Bouman and Deklerk, 1996).

McClintock (1984) suggested that the induction of genetic variability during stress might be a prerequisite for evolutionary adaptation. Furthermore, this may lead to activation of transposable elements (Peschke and Phillips, 1991).

1.3 *Petunia hybrida*

1.3.1 Classification, taxonomy, and occurrence

Petunia makes up the genus Petunia (Barton, 1970) of the family *Solanaceae* (Armeng, K.B., 1994). They are seasonal ornamental (Anupama *et al.*, 1999) often grown as annual (Hartmann and Kester, 1983; Christopher, 1994) or perennial (Jain, 1997; Barton, 1970) of the night shaded family. The leaves are alternate; the slender stems are weak causing the plant to sprawl.

Petunia flowers have five sepals, five petals fused to form a funnel-shaped corolla, five stamens, and solitary pistil (Encyclopedia, 2000) stamens are attached to the corolla tube (Barton, 1970) the fruit is two-celled, many seeded capsule (Encyclopedia, 2000), they are viscid with small entire leaves (Barton, 1970).

The genus, which contains about 35 species, is native to South America, chiefly Argentina and southern Brazil, is naturalized in Central America and Mexico (Encyclopedia, 2000).

Most of the present day garden petunias, *P. hybrid* appears to be derivatives of a single cross between *P. nyctaginiflora*, a species with white flowers, and the purple-flowered *P. integriflora*.

1.3.2 Importance of *Petunia hybrida*

This plant is popular for use in window boxes because its stems droop over the side's (Encyclopedia, 2000) hence the name balcony petunias (Encyclopedia, 2000; Armeng K.B, 1994).

Many strains of the common petunia have been developed by horticulturists including fringed and doubled flowers. Red, pink, purple and parti–colored flowers have been produced. Newer dwarf varieties with stronger stems, growing as allow mound, are popular edging plants for garden borders (Encyclopedia, 2000).

Petunia is of special interest in regard with meiotic segregation patterns and genome instability (Baird & Meagher, 1987; Corna and Maizonnier, 1983; Forster, 1996; Fransz, *et al.*, 1996; Koes, *et al.*, 1987; Mclean, *et al.*, 1990). Therefore, it remains a useful model system, particularly for studies of gene regulation and genome structure (Strommer, *et al.*, 1999).

Years of commercial breeding have produced varieties with defined genes of interest to biotechnology, (Mol, *et al.*, 1985;

Quat`trocchio, *et al.*, 1993; 1998; Huits, *et al.*, 1994; Solani, *et al.*, 1995; Bradley *et al.* 1998; Davies *et al.* 1998).

Several in vitro studies have been done on the plant *Petunia hybrida* including somaclonal variation (plant morphology, leaf morphology, flower color, flower shape, and leaf variation (Jain, *et al.*, 1998).

The influence of ethylene on shoot and root formation from petunia leaf explant was studied in cultures in test tubes placed in 51 glass jars. An ethylene absorbent caused a decrease of the number of shoots. On the other hand supplementing the cultures with ethylene (0.01-10ppm) caused a marked increase of the number of shoots without, however, any effect on the length and fresh weight (Kortessa, *et al.*, 1993).

Morphologically normal plants have been regenerated from petal protoplasts of petunia (*Petunia hybrida*) flower. Maximum protoplast yields from petal tissue were obtained within 2 days after anthesis. Protoplasts were cultured on modified Murashige and Skoge, M S medium. After culturing, protoplasts were found to reduce pigment density, and plastids development near the nucleus (Man and Sang, 1994).

The effect of environmental factors on the organogenesis of the plant *Petunia hybrida* was studied in vitro and it was found that

different controlling factors during the development of plantlets, and the interaction of these factors affects the organogenesis of the plant *Petunia hybrida* in vitro (Wang *et al.*, 1989).

The effect of explant size, configuration and duration of (BA) benzyladenine exposure to shoot organogenesis in *Petunia hybrida* leaf discs was determined. Leaf discs of different diameters end holes were tested. The smallest size and configuration for maximum shoot production and consistency of regeneration was a 10mm whole disc. The 13mm disc with a 5mm hole produced the most shoots. Exposure of leaf discs for (BA) containing medium for 4 days or less resulted in no shoots being formed. Exposure for 10 days or more induced shoot regeneration on all discs tested. A delay of more than 2 days after excision, before planting on benzeladenine containing medium, decreased shoot regeneration significantly (Beck and Camper, 1991).

1.3.3 Propagation of *Petunia hybrida*

Plant propagation is the multiplication of plant by both sexual and asexual means; *Petunia hybrida* is propagated sexually by seeds or asexually by production of new plant from the leaves, stems, or roots of a single parent plant. Asexual propagation is

advantageous when plants are well adapted to a particular environment.

Petunia is a relatively fast breeding dicot, that is easily maintained in the green house as pot plants (Christopher, 1994) and produce hundreds of seeds from a single pollination, (Mol *et al.*, 1985; Quattrocchio, *et al.*, 1993; 1998; Huits, *et al.*, 1994; Solani, *et al.*, 1995; Bradley *et al.*, 1998 and Davies *et al.*, 1998).

CHAPTER II

Materials and Methods

2.1 Regeneration experiments

2.1.1 Seed material

Mixed colored un-sterilized petunia seeds from Royal Flower Company were used in these experiments.

2.1.2 Sterilization of seeds

Seeds were enclosed in a white mesh and wrapped then were immersed in a Chlorax solution of 10% (0.52% sodium hypochlorite), then were rinsed three times in sterile distilled water for five minutes each time.

2.2 Medium preparation

Murashige and Skoog (MS) media was prepared by using MS salt in addition to 100mg/L myinistol, 30gm/L sucrose and 8gm/L agar. The pH was adjusted to 5.5-5.8, and then the medium was sterilized in the autoclave at 121^{0}C for 20 minute.

2.3 Seed plantation (Establishment of the plant material)

Petunia seeds were planted in 60 test tubes each containing 10-ml of MS basal medium (Table 3.1) 3 seeds for each test tube. Test tubes were incubated for four weeks in the incubator at 22^{0}C and day light 16 h under cool light at 50μ mol m^{-2} s^{-1} light intensity. To achieve large leaf size, shoots from the test tubes

were transferred into fresh MS media in plastic pots after the plastic pots were treated with 96% alcohol for 15 minutes.

2.4 Regeneration experiments

2.4.1 Regeneration from leaf disc explants

Under aseptic conditions Petunia leaves from the established plant material were sectioned into segments of 0.5 cm and transferred into 5cm diameter Petri dishes (3 segments for each plate) containing MS media with 8 different Naphthalene acetic acid (NAA) and Benzyl adenine (BA) hormonal combination (table 2.2). The 8 combinations were considered as treatments, the treatments were arranged in a completely randomized design. Each treatment was replicated 10 times (10 plates).

Plates were incubated in complete darkness for 2 weeks and transferred to the incubator under 22 $C^0 \pm 1$ and under 50μ mol m^{-2} s^{-1} light intensity for 16 h duration for another three weeks for callus and shoot initiation and development.

After one month, each plate was tested for the shoot regeneration, root and callus development. The experiment was repeated twice.

Table 2.1 Constitution of MS medium*

Compound	*concentration (mg/l)*
Major inorganic nutrients	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440.0
$MgSO4.7H_2O$	370.0
KH_2PO_4	170
Trace elements	
KI	0.83
H_3BO_4	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$NaMO_4.7H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$COCl_2.6H_2O$	0.025
Iron source	
$FeSO_4.H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organic supplements	
Myo-Inositol	100
Nicotinic acid	0.5
Pyridoxine –HCl	0.5
Thiamin-HCl	0.1
Glycine	2.0
Carbon source	
Sucrose	30000

*Murashing and Skoog (1962). The medium was supplemented with the following components; 30mg/l sugar; 8g/l agar; and the phytohormone (NAA) Naphthalene acetic acid and 6-benzelaminopurin (BA) at different concentrations as mentioned in the tables. The pH of the medium was adjusted to 5.7$\pm$0.1

Table 2.2 Hormone concentration* of NAA and BA used for callus production and shoot initiation

Treatment	Hormone concentrations (mg/l)	
	NAA	BA
1	0	0
2	0	0.5
3	0	1
4	0	2
5	0.2	0
6	0.2	0.5
7	0.2	1
8	0.2	2

The phytohormones NAA and BA were prepared as stock solution of 0.5 mg/ml by dissolving BA in 5ml HCL, and NAA in KOH and completing the volume with distilled water. The stock solution of the hormones was stored at 20°C.

2.4.2 Regeneration from shoot explant

Two liters of multiplication MS media containing (4.4 g MS powder, 30g sugar, 0.1 myoinositol) /L. Was divided into 8 beakers containing 250ml Ms Media with different hormonal concentration as seen in table (2.3). After adding the hormones, pH of each medium was adjusted in the range of 5.6-5.8. Two grams of agar were added for each beaker then boiled on a hot plate.

Each beaker was dispensed into 25 test tubes containing 10-ml of the media. Each group of test tubes was labeled and autoclaved for 30 min under 1.5 k.p /cm^2 and 121^{0}C. Two levels of NAA 0.0 and 0.1mg/l and 4 levels of BA, 0, 0.2, 0.4, 0.8mg/l were used in this experiment; the 8 combinations were considered as treatments. Each treatment was replicated 25 times the experiment was arranged as completely randomized design.

Shoots of about 2cm length from the establishment experiment were transferred into the hormonal combination media (one shoot per test tube) and incubated under 16 h day light (cool light) and 22C$^0\pm$ 1 in the incubator for four weeks.

After four weeks the number of shoots, length of shoots, number of leaves in each test tube (experimental unit) were recorded. Optimal hormonal combination was determined based on

the result of these variables. Analysis of variance was conducted followed by mean separation.

Table (2.3) Hormone concentrations of NAA and BA used for shoot multiplication

Treatment	NAA	BA
1	0	0
2	0	0.2
3	0	0.4
4	0	0.8
5	0.1	0
6	0.1	0.2
7	0.1	0.4
8	0.1	0.8

Concentration used was in mg/L

2.5 Somaclonal variation experiment

2.5.1 Plant material

Petunia hybrida seeds of the same source used in the regeneration experiment were planted in pots in the greenhouse and irrigated every other day for 2 months until sufficient growth was achieved.

2.5.2 Disinfection of the plant material

Lateral buds with a small piece of stem of 10 pink colored young plants was washed thoroughly with running tap water for 30 minutes, the buds were then placed in 250 ml of 10% chlorax containing 2 drops of Tween20 for 20 minutes, and washed 3 times

with sterile distilled water each time for 5 minutes, then the buds immersed in 96% ethanol for few seconds and left to become dry.

The buds were trimmed to remove bleached damaged tissues then transferred to test tubes containing MS basal media. The media were supplemented with either Benlate (fungicide) or Gentamycin sulfate (Antibiotic), or by both Benlate and Gentamycin sulfate. 20 test tubes for each treatment were used (Table 2.4), 20 tubes were used as a control.

Test tubes were transferred to growth chamber under 25°C±1 and 16h daylight; cool light for growth. After two weeks test tubes for each treatment were tested for contamination percentage and shoot length.

Table 2.4 MS media with different combinations of Gentamycin sulfate and Benlate

Treatment	GS	BE
1	+	+
2	–	+
3	+	–
4	–	–

***Concentrations used was 30mg/l from each material**
*** Treatment number 4 was used as a control**

2.6 Shoot Regeneration

Growing uninfected shoots of 2 cm length were transferred from the previous experiment to test tubes each containing 10 ml

of shoot regeneration media (MS media supplemented with 0.4 mg/l BA) incubated in the growth room under 16 h day light, 25^0C±1 for four weeks.

2.7 Root induction and acclimatization

Regenerated shoots from the previous experiment were sectioned for 2-cm length immersed in 6-Indole butyric acid (IBA) hormone powder and transferred into pots containing sterile rooting media (2:1 mixture of sterile peatmoss and vermiculite).

Shoots were irrigated by sterile distilled water incubated for three weeks in the growth room under 16 h days light in 25^0C ±1 after three weeks the number of shoots which produce roots was counted.

Rooted shoots, were transferred gently to larger pots containing 2:1 ratio of peatmoss and vermiculite, then transferred to the green house and irrigated every other day for morphological evaluation.

Leaf morphology, flower color, flower shape was compared with the original plant.

CHAPTER III

Results

3.1 Regeneration experiment

3.1.1 Seed plantation

After 3 weeks, all of the seeds germinated successfully onto the basal medium (Fig 4.1), they continued to grow on this media for one month. All of the seedlings were clean (no contamination was observed).

3.1.2 Leaf explant

3.1.2.1 Callus initiation

After 4 weeks of incubation; callus growth was significantly higher on solid medium containing 0.8 mg/l BA and 0 mg/l NAA (Table 4.1). The second medium, which produced callus, was containing 0.4 mg/l BA and 0 mg/l NAA. There was a significant interaction between BA and NAA on callus formation.

3.1.2.2 Shoot regeneration

Both BA and NAA exhibited a significant effect on shoot regeneration in both experiments. The highest shoot percentage (20) was obtained from leaf explant on medium containing 0.8mg/l BA and 0 mg/l NAA (Table 4.2) and (Fig 4.2). This treatments, however, was not different in shoot percentage from the medium containing 0.4 mg/l BA in presence of 0.1 mg/l NAA. No growth of shoots was achieved on the other combinations. A significant

interaction between BA and NAA was also exhibited on shoot regeneration.

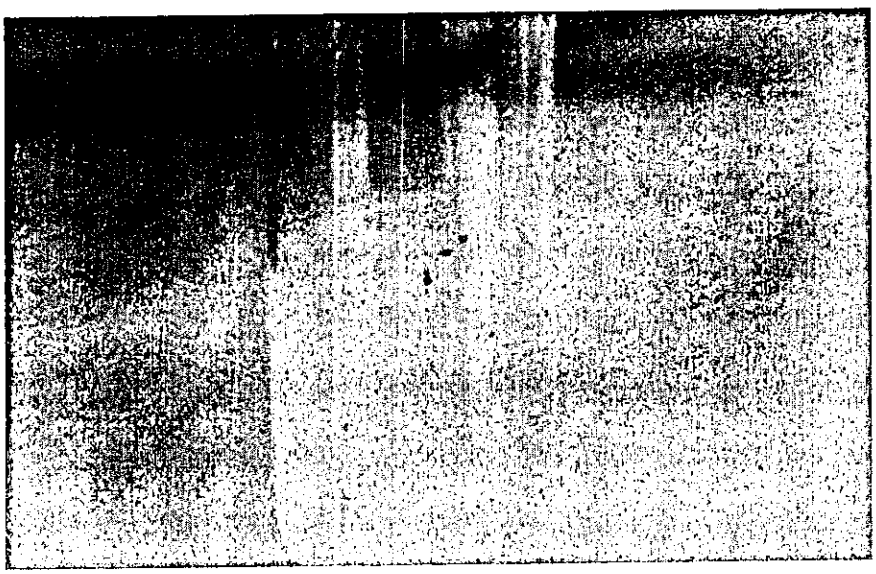

Fig 3.1 Growing *Petunia hybrida* seeds on MS basal media

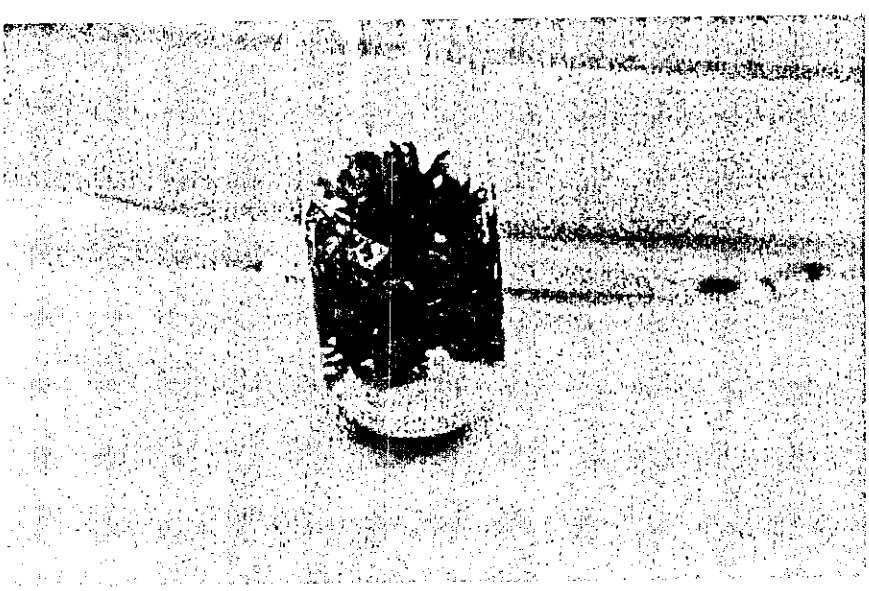

Fig 3.2 Regenerated shoots from *Petunia hybrida* (leaf explant) planted on MS media containing 0.8 mg/l BA

Table 3.1 The effect of different levels of BA and NAA on callus initiation percentage of *Petunia hybrida* (leaf explant)

	BA (mg/l)							
	Exp 1				Exp2			
NAA(mg/l)	0	0.2	0.4	0.8	0	0.2	0.4	0.8
0.0	0.0c	0.0c	60b	100a	0.0c	0.0c	60b	90a
0.1	20c	0.0c	10c	22c	20c	0.0c	10c	22c

Number followed by the same letter or letters are not significantly different at 5% level according to (Duncan,s Multiple Range Test) (DMRT)

Table 3.2 The effect of different levels of BA and NAA on adventitious shoot regeneration percentage of *Petunia hybrida* (leaf explant)

	BA (mg/l)							
	Exp 1				Exp2			
NAA(mg/l)	0	0.2	0.4	0.8	0	0.2	0.4	0.8
0.0	0.0b	0.0b	0.0b	20a	0.0b	0.0b	0.0b	20a
0.1	0.0b	0.0b	20a	0.0b	0.0b	0.0b	20a	0.0b

***Number followed by the same letter or letters are not significantly differ at 5% level according to (Duncan,s Multiple Range test) (DMRT).**

3.1.2.3 Shoot number

Both BA and NAA didn't exhibit a significant effect on shoot number in both experiments. However, the highest shoot number was achieved from leaf explant growing on medium containing 0.4 mg/l BA and 0.1 mg/l NAA (Table 4.3).

The medium, which contains 0.8 mg/l BA, and 0 mg/l NAA gave lower shoot number but the other combinations gave no shoots.

3.1.2.4 Root percentage

There was no significant interaction effect between BA and NAA on root initiation, in addition BA did not show a significant effect on root initiation. Therefore the effect of NAA was presented alone.

Medium, which contains 0.1mg/l NAA, gave the highest root percentage in both experiments (Table 4.4).

3.1.3 Shoot explant

3.1.3.1 Shoot number

Both BA and NAA exhibited a significant effect on the average number of regenerated shoots without interaction between

the growth regulators, therefore, the effect of each growth regulator was presented separately.

Table 3.3 The effect of different levels of BA and NAA on the average shoot number produced from regenerated explants of *Petunia hybrida*

	BA(mg/l)							
	Exp 1				Exp2			
ANN(mg/l)	0	0.2	0.4	0.8	0	0.2	0.4	0.8
0.0	0.0b	0.0b	0.0b	0.6a	0.0b	0.0b	0.0b	1.1a
0.1	0.0b	0.0b	1.7a	0.0b	0.0b	0.0b	1.7a	0.0b

Number followed by the same letter or letters are not significantly differant at 5% level according to (Duncan,s Multiple Range test) (DMRT)

Table 3.4: The effect of NAA on the adventitious root percentage of *Petunia hybrida*

Concentration of NAA used (mg/l)	Rooting %	
	Exp1	Exp2
0.0	4b	0b
0.1	32.5a	20a

Number followed by the same letter or letters are not significantly different at 5% level according to (Duncan,s Multiple Range test) (DMRT)

3.1.3.1.1 Effect of BA on the average number of regenerated shoots

After 4 weeks of incubation the highest shoot number (7.8) (Fig 4.3) was achieved with the medium containing 0.8 mg/l BA (Table 4.5). However, the three other levels of BA including the control resulted in similar shoot number without significant difference among them. The lowest average shoot number (2.89) was obtained with the control.

3.1.3.1.2 Effect of NAA on number of regenerated shoots

The highest shoot number (6.2) was obtained on medium containing 0.1 mg/l NAA (Table 4.6). Medium containing 0.0mg/l NAA showed lower number of regenerated shoots.

4.1.3.2 Effect of BA and NAA on the average length of the regenerated shoots

Both BA and NAA exhibited a significant effect on the average shoot length with interaction. The highest shoot length (4.63) was obtained on medium containing 0 mg/l BA and 0.1 mg/l NAA (Table 4.7). But this did not differ significantly from medium containing (0.2, 0) and (0.4, 0) mg/l of both BA and NAA, respectively.

The lowest average shoot length was obtained with medium containing 0.1 and 0.2 mg/l of NAA and BA respectively.

Fig 3.3 The highest shoot number obtained on MS media containing 0.8 mg/l BA

Table 3.5 The effect of different levels of BA on the number of regenerated shoots

BA(mg/l)	Average number of shoots
0.0	2.89b
0.2	5.22b
0.4	3.9b
0.8	7.8a

*Number followed by the same letter or letters are not significantly different at 5% level according to (Duncan,s Multiple Range test) (DMRT)

Table 3.6: The effect of different levels of NAA on the number of regenerated shoots

NAA(mg/l)	Average number of shoots
0.0	3.9b
0.1	6.2a

*Number followed by the same letter or letters are not significantly different at 5% level according to (Duncan,s Multiple Range test) (DMRT)

3.1.3.3 Leaf number

Similar significant effect of both BA and NAA was exhibited on the average leaf number per test tube.

The highest leaf number (25) per test tube was obtained from the medium containing 0.8 mg/l BA and 0 mg/l NAA (Table 4.8).

Table 3.7: The effect of different levels of BA and NAA on the average length (cm) of the regenerated shoots

	BA mg/l			
ANN(mg/l)	0	0.2	0.4	0.8
0	3.00bc	4.00a	4.60a	3.80ab
0.1	4.63a	2.33c	3.20c	3.40b

***Number followed by the same letter or letters are not significantly different at 5% level according to (Duncan,s Multiple Range test) (DMRT)**

Table 3.8 The effect of BA and NAA on the average leaf number per test tube

	BA mg/l			
ANN(mg/l)	0	0.2	0.4	0.8
0	15.20b	16.40b	12.40bc	25.00a
0.1	9.00cd	5.75d	6.80d	5.60d

***Number followed by the same letter or letters are not significantly different at 5% level according to (Duncan,s Multiple Range test) (DMRT)**

3.2 Somaclonal variation experiment

3.2.1 Plant material

After two months of incubation, sufficient growth of lateral buds was achieved.

3.2.2 Contamination experiment

After two weeks of incubation, medium containing 30 mg/l gentamycin sulfate and 30 mg/l benlate resulted in the lowest contamination percentage (18%), and was the second best medium for shoot growth (4.55 cm) (Table 4.9). When gentamycin was used alone the highest shoot length (5.9cm) was achieved, but the contamination percentage was higher than that obtained on medium containing both gentamycin and Benlate.

The highest contamination percentage (69%) was obtained in the control media, and the shortest average shoot length (2.45cm) was obtained when Benlate was used alone.

Table 3.9 The effect of Gentamycin sulfate and Benlate on the contamination percentage and the average shoot length of lateral buds of *Petunia hybrida*

Treatment	Contamination %	Shoot length (cm)
1. Gentamycin & Benlate	18 b	4.55 b
2. Benlate	63 a	2.45 c
3. Gentamycin	28 b	5.90 a
4. control	69 a	2.77 c

***Number followed by the same letter or letters are not significantly different at 5% level according to (Duncan,s Multiple Range test) (DMRT)**

3.2.3 Acclimatization experiment

After three weeks of incubation on the rooting medium 80% of the shoots produced roots (Fig 4.4), the rooted plants continued to grow in the green house (Fig 4.5).

3.2.4 Morphological evaluation

3.2.4.1 Leaf variation

Among sixty growing plants in the green house (Fig 4.6) leaves were inspected for morphological variation. The shape of the original plant leaves (normal) was ovate (Figs 4.7a, b), another two new forms of leaves were obtained, the shape of (form 1) was orbicular and the shape of (form 2) was elliptic.

3.2.4.2 Flower variation

Among 40 plants that continued their growth in the green house until flowering, three new colors were shown (Fig 4.8). 5 plants produced purple colored flowers, 10 were dark pink, and 5 were violet, while the others were similar to the original color (Fig 4.9).

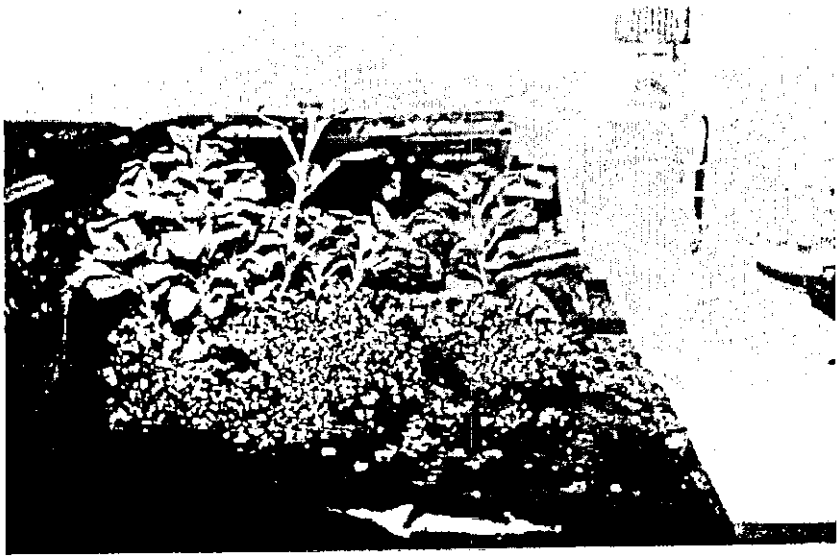

Fig 3.4 *Petunia hybrida* shoots that produced roots after IBA treatment

Fig 3.5 Regenerated *Petunia hybrida* shoots into pots containing sterile rooting media

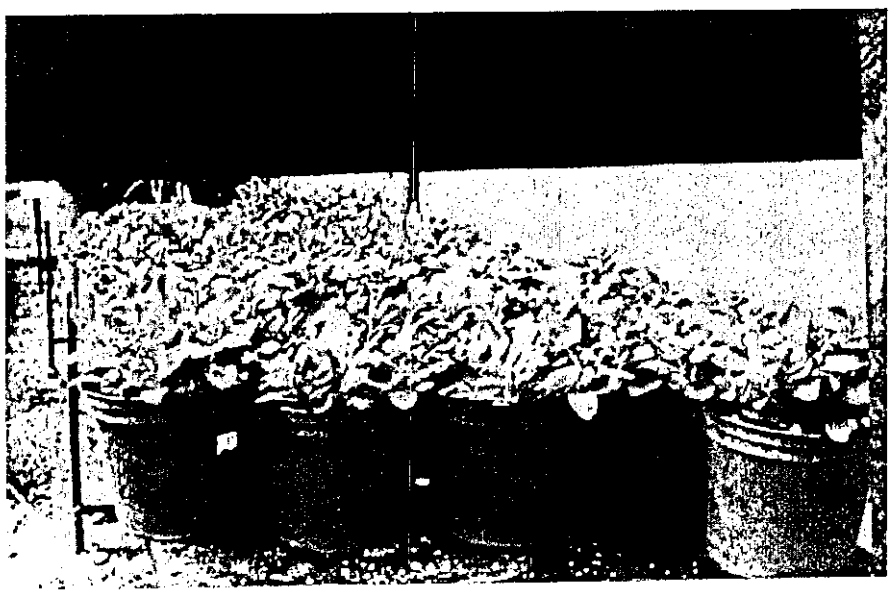

Fig 3.6 Successfully regenerated *Petunia hybrida* plants transferred to the green house for morphological evaluation

Fig 3.7a Different forms of leaves of *Petunia hybrida* plants

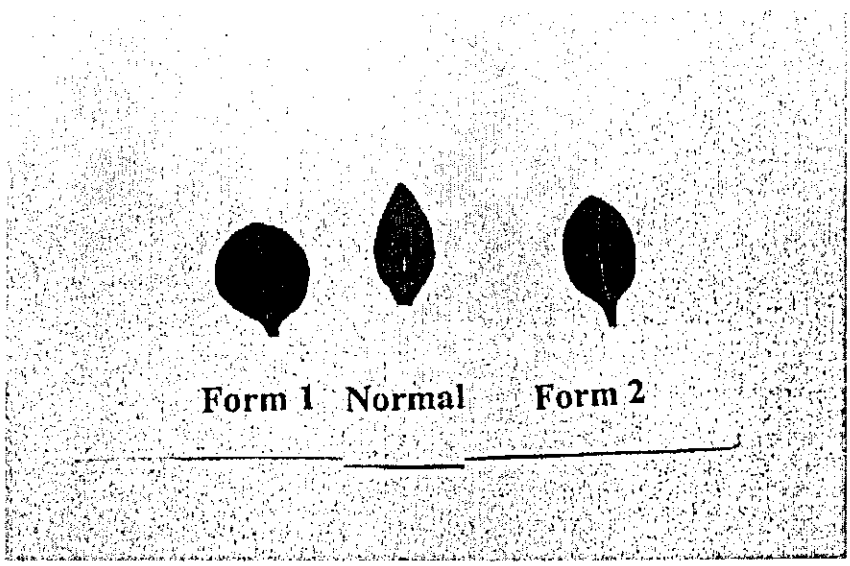

Fig 3.7b Three forms of *Petunia hybrida* leaves, Normal (ovate) is the form of the original plant, form 1 (orbicular) and form 2 (elliptic) are new forms

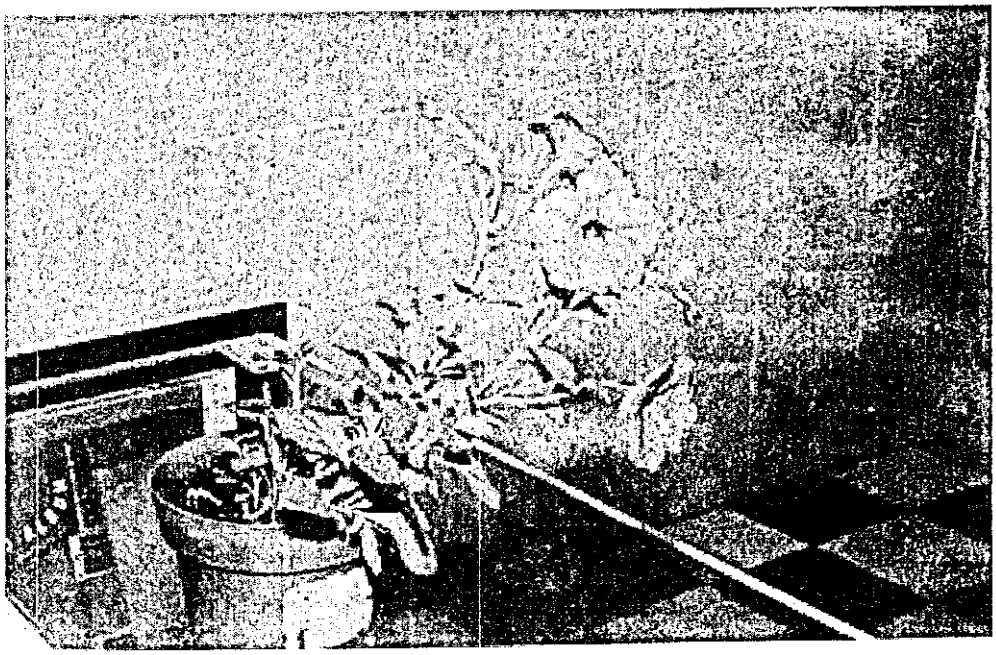

Fig 3.8 The original *Petunia hybrida* plant with pink colored flowers, which was used as a source of plant material

Fig 3.9 Different colors of *Petunia hybrida* flowers, the original (pink), the new forms are violet, purple, and light pink

CHAPTER IV

Discussion

4.1 Seed plantation

From the previous experiments, it was proved that seeds are a good starting material for the production. High germination percentage was achieved on agar medium without growth regulators. This result agreed with a previous work (Dixon, 1985).

4.2 Regeneration

4.2.1 Leaf explants

Similar to the findings of (Jean *et al*, 1990), the result of the present study demonstrated that BA is important for callus initiation because when BA used alone, at 0.8 mg/l, callus initiation was the highest, when used alone at lower concentration 0.4 mg/l, callus growth was lower. But no growth was observed when free BA media was used.

Organogenesis is mainly controlled in part by the balance between auxin and cytokinin (Flick *et al.*, 1983; Skoog and Miller, 1957; Tran, 1981). The elucidation of hormone action is intricate because organogenesis is a complex response, which includes mitosis, polarization, primordium formation, and meristem development (Reinert *et al.*, 1977; Thorpe, 1980); each of these processes has likely its own hormonal control. Moreover, growth substances may also induce the competence of tissue to respond to

further developmental signals (Christianson and Warnick, 1985; Christianson and Warnick, 1988), cells often respond differently at different developmental stages, and interactions between auxin and cytokinin signaling pathways my occur (Shi *et al.*, 1994).

The inclusion of cytokinin in culture media enable callus to be induced from a wide range of plant species but more importantly to initiate multicellular meristematic regions capable of differentiation into organized structures (Lowe *et al.*, 1996). But no regeneration for *Petunia hybrida* calli subcultured on low cytokinin media. (Renaudin *et al.*, 1991)

NAA might have an inhibitor effect for BA because as observed by (Jean *et al.*, 1996) callus growth was dramatically reduced when 0.1 mg/l NAA is added in the media.

4.2.2 Shoot production

In vitro adventitious shoot formation on Petunia explants was promoted by adding BA to basal media (Daykin *et al.*, 1976. Roa, 1973; Read, 1979) or by BA pretreatment of leaf segments prior culture (Economou and Read, 1980) or according to (Thorpe *et al.*, 1991) that cytokinin alone is sufficient for shoot formation.

Similar to the findings of the present study it is apparent in petunia that optimal adventitious shoot formation on leaf explants

is promoted by an auxin-cytokinin combination. According to the regulatory model of cytokinin-auxin ratio demonstrated by Skoog and Miller (Skoog and Miller, 1957).

However, the variability among petunia cultivars in shoot production in vitro observed when cytokinin alone was used (Jainand De klerk, 1998) and the present findings of enhanced shoot production by incorporating NAA in the medium suggest that a better balance of cytokinin–auxin ratio is achieved by applying both hormones in the appropriate amounts. Rao, *et al.*, 1973, observed the failure of 0.2 mg/l BA and 0.1-mg/l NAA combination to induce shoots. Perhaps the high concentration of NAA inhibited bud initiation.

This would agree with the present study findings of depressed shoot production with the highest NAA concentration tested, which was less than that used by (Rao *et al.*, 1973).

In general, other factors may affect number of shoots per explant, Increased duration of exposure to BA resulted in higher shoot number (Auer *et al.*, 1992). Changing the nutrient medium can also dramatically alter the percentage of explants forming adventitious shoots and/or number of adventitious shoot buds (Gomez and Segura, 1994).

4.2.3 Root percentage

The findings of this study were similar to those of (Brown and Charlwood, 1986; Fosket, 1976), they demonstrated that organogenesis can be induced or halted by manipulating the ratio of auxin to cytokinin in the medium. For example a high ratio of auxin to cytokinin can induce the formation of roots in dicotyledonous callus (Warren, 1991) also reported that the ratio of auxin to cytokinin influence the balance between root and shoot formation, high auxin relative to cytokinin favors root formation and the converse situation favors shoot formation, while cytokinins inhibit root formation (Flick *et al.*, 1983; Skoog and Miller, 1957; Tran, 1981).

4.3 Shoot explants

The number of adventitious buds and shoots produced by explants may be easily regulated by varying the concentration of BA in the culture medium (Pollard and Walker, 1990).

By experiments, the results of this research were in correspondence with Douglas, who in 1985 demonstrated that the influence of growth regulators on shoot formation in explants is in increased number of shoots produced in explants of various lengths (Douglas, 1985).

In 1978, Read found that red light (which frequently produce cytokinin- like effect) increased the branching of Petunia stock plants and also increased shoots produced from explants *in vitro* (George & Sherrington, 1984).

The exposure of the explants to BA containing medium increased number of explants produced per explant dramatically (Auer *et al*, 1992). Cytokinin as a growth regulator in the culture medium increase the number of shoots produced in explants of various lengths (Douglas, 1984).

4.4 Contamination Experiment

Gentamycin reduced the contamination rate dramatically to 28% when used as an anti bacterial agent in the media (Spencer *et al*., 1980).

From the present study, it can be noted that Gentamycin increases the growth rate of *in vitro* shoots 5.09 cm, Like the findings of (George and Sherrington, 1984) some antibiotics have been noted to increase the growth rate of cultured tissues some times in spectrum way.

When benlate was supplied to the medium, the contamination percentage was reduced; the growth rate was also affected. Some plant tissues have internal contaminants that can not be removed

by standard techniques (Pillai and Hildebrandt, 1969; Abu El-Nil and Hildebrandt, 1971). The addition of benlate to the medium, prove to be effective in reducing fungal contamination; however, benlate can affect subsequent plant development adversely (Abu El-Nil and Hildebrandt, 1976.; Abu El-Nil and Hildebrandt, 1973).

4.5 Somaclonal variation

The result of the present study demonstrated that new phenotypes from *Petunia hybrida* could be produced by somaclonal variation through in vitro multiplication. Leaf morphology was obtained and two new forms were produced, form 1 orbicular and form 2 elliptic. (Deklerk, *et al.*, 1990); Bouman and Deklerk, 1996) examined the extent of variability of leaf shape in ornamental plants. A large increase of variability was observed after regeneration from non-organized callus. Variation was also observed among flower color; three new colors were observed purple, dark pink and light pink.

Variation has been observed in ornamental plants with respect to leaf shape, flowers color. Flower color has long been one of the target traits in conventional breeding. The main pigments contributing to flower color are the flavonoids, imparting yellow, red, purple and blue colors, and carotenoids imparting yellow to

orange colors. The color of the pigments strongly depends upon the pH within the cell (Jain and Deklerk, 1998).

Petal pigmentation in most flowers, including petunia, is a function of anthocyanin content (Martin and Gerats, 1993). Anthocyanin content is affected by many factors such as temperature, for instance, it was found that lower growing temperature enhanced the pigmentation, size, and weight of petunia flowers (Shvarts *et al.*, 1997). Martin and Gerats, 1993 demonstrated that flowers produced at 17/12°C contained more than twice the anthocyanin of flowers produced at 32/27°C (Martin and Gerats, 1993). The synthesis of flavonoid pigments in petals of most flowers is accompanied by tissue growth and is under developmental control (Martin and Gerats, 1993), and this process has been studied intensively in petunia flowers. At least two factors, gibberellin and light, are involved in the regulation of anthocyanin synthesis and growth during petunia flower development. The induction of pigmentation by these factors results from the activation of expression of anthocyanin biosynthetic genes, including phenylalanine amonia-lyase (*pal*), chalcone synthase (*chs*), chalcone-flavanone isomerase (*chi*), dihydroflavonol 4-reductase (*dfr*) and anthocyanidin synthase(*s*) (Weiss and Halvy, 1989; Weiss *et al.*, 1993; Weiss, 1995).

Slabaeck-Szweykowska in 1952 carried studies on the effect of various chemical and environmental factors on anthocyanin formation. It was found that increased sucrose levels in the medium resulted in more anthocyanin development (Slabaeck-Szweykowska, 1952).

4.6 Summary and conclusion

1.Shoot explants were better than leaf section for shoot regeneration.

2.Cytokinin (BA) at 0.4 mg/l and Auxin (NAA) at 0.1 mg/l was the best for shoot regeneration from leaf explants.

3.BA at 0.8 mg/l and NAA at 0.1 mg/l were the best for stem regeneration from shoot explants.

4.Gentamycine sulfate at 30 mg/l with benlate at 30 mg/l incorporated in the MS medium were the best for reducing contamination.

5.Somaclonal variation can be used as a source of selectable heritable variability, however further work and follow up of second generation is necessary.

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الملخص باللغة العربية

إكثار نبات بيتونيا *Petunia hybrida* داخل الأنابيب وأثر ذلك على التغيرات الشكلية

أنس يوسف أبو ريا

تم في هذه التجارب دراسة اثر مستويات مختلفة من ألا كسين (NAA) والسايتوكاينين (BA) على إكثار سيقان نبات بيتونيا هايبريدا، لقد تم تنمية السيقان الناتجة من طرق التكاثر المختلفة لملاحظة التغيرات الشكلية الناتجة عن طريق التكاثر. تم زراعة بذور نبات بيتونيا *Petunia hybrida* داخل أنابيب على بيئة موراشيج وسكوج (MS). تم استخدام النباتات النامية في الأنابيب مصدرا لتجارب الإكثار. استخدام أربع تراكيز من BA (0 ؛0.1 ؛ 0.4 ؛ 0.8) ملليجرام /لتر وتركيز بين من NAA (0 ؛0.1) ملليجرام /لتر .كما تم استخدام مصدرين من النباتات في هذه التجارب من مقاطع من الأوراق ومن السيقان. أظهرت التجارب التي تم استخدام مقاطع من الأوراق نسبة إكثار متدنية، وأعلى نسبة إكثار سيقان تم الحصول عليها على بيئة (MS) تحتوي على (0.4) ملليجرام /لتر (BA) و (0.1)ملليجرام /لتر (NAA). أما في التجارب التي تم استخدام السيقان كمصدر للنباتات فيها تمايز عدد أعلى من السيقان عند استخدام (0.8) ملليجرام /لتر (BA) مع (0.1) ملليجرام /لتر (NAA) أما بالنسبة للتغيرات الشكلية فقد تم استخدام براعم من نباتات وردية اللون تم تربيتها في البيت البلاستيكي .تم تعقيم هذه البراعم ثم زراعتها على بيئة MS تحتوي على 30 ملليجرام /لتر سلفات الجنتامايسين (مضاد حيوي) و30 ملليجرام /لتر بنليت ، بعد نمو البراعم تم نقل السياقان على بيئة إكثار

السيقان (M S) تحتوي على (0.8) ملليجـرام /لتـر (BA) مـع (0.1) ملليجرام /لتر (NAA). تم تجذ ير وأقلمة النباتات للتقيـيم حيـث نـتج شكلين جديدين من الأوراق (orbicular and clliptic) وثلاثـة ألـوان مختلفة من الأزهار(بنفسجي، وردي، وردي فاتح).